# THE SEPARATION OF PHYTOCHEMICAL COMPOUNDS OF *SPICA PRUNELLAE* AND PERICARPIUM OF *CITRUS RETICULATAE* BY USING A REVERSE PHASE HPLC METHOD

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# CERTIFICATE

This is to certify that the dissertation entitled "The separation of phytochemical compounds of *Spica prunellae* and pericarpium of *Citrus reticulatae* by using a reverse phase HPLC method" is the bonafide record of research work done by Mr. Gan Chin Chear during the period from July 2008 to October 2008 under my supervision.

# ABSTRACT

A reversed-phase HPLC-UV method was developed for the separation of phytochemicals in water, n-hexane, and methanol extracts of two of the very common used Chinese herbal medicine, *Spica prunellae* and pericarpium of *Citrus reticulatae.* The phytochemical components of these extracts were separated by RP-HPLC on a C-18 column and adjusting mobile phase (acetonitrile: methanol; 40: 60 v/ v), flow rate at 0.8 mL/min.

The identification of phytochemicals separated by RP-HPLC with UV-detection at 280 nm and 320 nm is based on the comparison of peak retention times of some previously published works. However, this needs to be confirmed by the use of standard samples under the same experimental condition.

# ABSTRAK

Satu kaedah HPLC-UV fasa terbalik telah direka dan diguna untuk pemisahan konstituen fitokima dalam ekstrak air, n-Heksana, dan metanol daripada dua herbal tradisional cina yang biasa digunakan, iaitu *Spica prunellae* dan kulit *Citrus reticulatae.* Komponen fitokimia dalam ekstrak-ekstrak ini dipisahkan dengan mengunakan kaedah RP-HPLC bersama medan C-18 dengan penyesuaian fasa mobile (asetonitril: metanol; 40: 60 isipadu / isipadu), dan laju aliran fasa mobile pada 0.8 mL / minit.

Pengenalpastian konstituen fitokimial yang dipisahkan oleh RP-HPLC dengan UV-deteksi pada 280 nm dan 320 nm adalah dilakukan berdasarkan pada perbandingan puncak masa retensi daripada beberapa hasil kajian yang diterbitkan sebelumnya. Namun, Pengenalpastian ini perlu disahkan dengan penggunaan sampel piawai pada keadaan eksperimen yang sama.

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# CHAPTER ONE
# INTRODUCTION

## 1.1 Traditional Chinese Medicine

Traditional Chinese Medicine (TCM) is a range of medical practices used in China for more than 4000 years. It is also recognized as a popular complementary and alternative medicine in Western countries because to it is generally extracted from natural products without artificial additives which creates mild healing effects and causing less side effects (Lukman *et al.*, 2007). Even though the TCM was popular in other countries, the pharmacological properties of many Chinese herbs were only well documented in China. There is a lack of information on how Chinese herbs are actually used in practice outside China (Teng *et al.*, 2008).

In general, the composition of herbal medicines is complex and active components are rarely identified. In addition, the quantities of active compounds and marker compounds in herbal medicines are dependent on intraspecies variability, environmental conditions, harvest period, storage time, and processing method (Lee *et al.*, 2009). Furthermore, the effects of TCM toward a person are described based on the philosophical frameworks such as the theory of Yin-Yang and five elements, the human body meridian systems and the Zang Fu theory (Lukman *et al.*, 2007). The exact active ingredient (s)

that is responsible to cure the disease is not clearly known for many Chinese herbs and biochemical pathway of the certain herbal medicine as well.

Some of the Chinese herbs are very economical and Chinese population has been using them to make the herbal tea or condiment regularly (Wang *et al.*, 2008, Reid; 2001; Dai and Liu, 1999; Ody, 2000). However, the phytochemical content of the herbal was unclear. The population practice on the TCM as common food or drink may have some physiological effects on body whether it is giving benefit or in another way.

In this project, two of the most common Chinese herbs: *Spica prunellae* and *Citrus reticulatae* have been selected for qualitative and/ or semi-quantitative identification of their major phytochemicals.

## 1.2 *Spica prunellae*

*Spica prunellae* also known as *Xia-ku-cao* or *self-heal*, is a Chinese Traditional medicine herb being used for the traditional medicine practice. The scientific name for *Spica* prunellae plant is known as *Prunella vulgaris* (Hou & Jin, 2005). *S. prunellae* is the spike or whole plant of the perennial herb. This herb can be found in many places in Europe and Asia (Keys, 1976; BeijingXueYuan, 2005; WHO, 1989).

*P. vulgaris* is a low or sprawling perennial herb about 45 cm tall. Its inflorescence/ spike has a compact head which is provided with broad and

persistent bract. In China, the herb is collected in summer when the spike becomes brownish-red, removed from foreign matter, and dried under the sun (Hou & Jin, 2005). The spike is bitter and pungent (Keys, 1976).

This herb is popular among the Chinese population as the constituent of herbal tea. In Malaysia, many cafés and restaurants serve the herbal tea with this herb as the main constituent. Besides, *S. prunellae* also is used for the preparation of other decoction (Ody, 2000) and herbal drink recipe, such as *tien teng gout eng yin*, which is helpful for the people who had high blood pressure (Reid, 2001).

It is traditionally believed that this herb is able to clearing up liver-heart, purging fire, subduing swellings, and resolving hard lumps (BeijingXueYuan, 2005; Ody, 2000). Its nature property is cold, which is the reason why it acts on liver and gallbladder meridians to clean the "liver fire" or "gan hao" in mandarin (Hou & Jin, 2005; Ody, 2000). This *S. prunellae* possess and used for following purpose:

1. Antibacterial (Li, 2002)
2. Antipyretic (Li, 2002; Keys, 1976)
3. Cardiac tonic (Li, 2002)
4. Diuretic (Li, 2002; Keys, 1976)
5. Anticancer (Li, 2002)
6. Gout (Keys, 1976)
7. Throat relaxing (Wheelwright, 1974)

8. Hypertension (WHO, 1989; Hou & Jin, 2005)
9. Skin inflammation (WHO, 1989)
10. Anti-rheumatic and detoxicant (Hou & Jin, 2005)
11. Dry cough (WHO, 1989)

The recommended dose of this herb is 5-8 g per serving (Keys, 1976; WHO, 1989).

## 1.3 Pericarpium of *Citrus reticulatae*

Pericarpium of the *Citrus reticulatae* also known as chen-pi, is one of the Chinese Traditional medicine herbs, which has been used for the traditional medicine practice. It is the rind of the King orange or mandarin orange fruit. The fruit is nearly spherical, orange or reddish, flattened at the two ends, zest bruised but not warty (Keys, 1976). This fruit (used as herbal medicine purpose) can be found mainly in the Southeastern China: Guangdong, Sichuan, Fujian, Jiangxu, Zhejiang, Hunan, and Yunnan; in Vietnam (Keys, 1976; Hou & Jin, 2005).

This herbal medicine is harvest from the fresh unripe or ripe fruit, and dried for usage (Ody, 2000). According to WHO, pericarpium of the *C. reticulatae* is officinal, and its odour is aromatic while the taste is pungent and bitter (WHO, 1989). To reduce the bitter taste, older rinds are preferred because aging causes breakdown of the bitter substances (Hyatt, 1990).

Traditionally, this herb is used as one of the component in the herbal tea, syrup, tonic or decoction (Dai & Liu, 1999; Reid, 2001; Ody, 2000). In some families, peel of tangerine is extensively added to food as condiments (Wang *et al.*, 2008). It is believed that the rind of the fruit is warm in nature and able to promote the flow of the vital energy or "Qi", clears the phlegm, and acts on the spleen and lung meridians (Dai & Liu, 1999; Hou & Jin, 2005). This herb is used for the following purposes:

1. Stomachic and digestant (Hyatt, 1990; Keys, 1976; WHO, 1989; Dai and Liu, 1999; Li, 2002; Ody, 2000)
2. Expectorant (Keys, 1976, Li, 2002)
3. Antitussive (productive cough) (Hyatt, 1990; Keys, 1976; WHO, 1989; Li, 2002; Ody, 2000)
4. Antiemetic (Keys, 1976; Li, 2002)
5. Alcoholic intoxication (Hou & Jin, 2005)
6. Diuretic (Hyatt, 1990)
7. Anti-belching (WHO, 1989)

The advisable dose of this herbal medicine is between 3-9 g per serving (Keys, 1976; WHO, 1989; Hou & Jin, 2005).

According to the well known pharmacopeia in China, "Pen Ts' Kang Mu" (A.D. 1596), the pericarpium of the *C. reticulatae* is already been used as a herbal medicine since 500 years ago in China, and it's components were documented as 22% of fruit constituents, 3.8% of essential oil, 9% of pentosans, 0.8% of ash, and some vitamin A and B (Read, 1982).

However, with the modern technology of this era, the components of the herbal medicine can be separated by using more advanced technique, such as chromatography and then, the constituents in the herbal medicine can be further identified (Liang *et al.*, 2004).

## 1.4 High Performance Liquid Chromatography (HPLC)

High-pressure liquid-solid chromatography (HPLC) is rapidly becoming the method of choice for separations and analysis in many fields.

The HPLC separation is achieved by injecting the sample dissolved in solvent into a stream of solvent being pumped into a column packed with a solid separating material. The interaction is a liquid-solid separation. It occurs when a mixture of compounds dissolved in a solvent can either stay in the solvent or adhere to the packing material in the column (McMaster, 2007).

Currently, HPLC is a popular method for the analysis of herbal medicines because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. In general, HPLC can be used to analyze almost all the compounds in the herbal medicines by controlling the flow rate, mobile phase and other factors (Liang *et al.*, 2004).

This popular technique still continues its evolution, particularly in the life sciences and biotechnology area, and has became the most widely used analytical technique (Ettre, 2002). One of the most common type of the HPLC technique, reverse-phase HPLC (RP-HPLC), which is reversed-phase (RP)

columns for the analytical separation of various herbal medicines (Liang *et al.*, 2004), such as, *Psoralea corylifolia* (Zhao L. *et al.*, 2005); *Radix Aconiti Lateralis Preparata* (Xie et al., 2008); *Ganoderma lucidum* (Tang et al., 2006); *Tectona grandis, Shilajit, and Valeriana wallachi* (Srivastava, 2008).

In the present work, various extracts of the *C. reticulatae* and *S. prunellae* were tried to separate using RP-HPLC and identify their constituents.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 *Spica prunellae*

Hou & Jin (2005) stated that *S. prunellae* contains triterpeneniods, flavonoids, sterol glycosides, and coumarins. The triterpene compounds include ursolic acid and betulinic acid. The flavonoids include delphinidine, cyaniding, and rosmarinic acid. The sterol glycosides include beta-sitosterol-beta-D-glucose. Other ingredients are alkaloids, oleanoic acid, rutin, hyperoside, caffeic acid, tannin, volatile, and vitamin A, C and K (Hou & Jin, 2005). The extensively studied constituent in *S. prunellae* is rosmarinic acid.

For antioxidant effect of the *S.prunelae*, some *in vitro* studies are reported in literature. In a study using human keratinocytes, the cells was exposed to 10–30 J/ $cm^2$ ultraviolet radiation A (UVA) and then treated with an extract of *S. prunellae* (1–75 mg/ L) or rosmarinic acid (0.9– 18 mg/ L) for 4 hours. The results suggest that *S. prunellae* extract and rosmarinic may offer protection against UVA-induced oxidative stress and may be beneficial as a supplement in photoprotective dermatological preparations (Psotova *et al.*, 2006).

In another *in vitro* study, the aqueous ethanol extract of this herb and its component rosmarinic acid were tested on LPS-induced oxidative damage and inflammation in human gingival fibroblasts. Both of the *S. prunella* extract and rosmarinic acid reduced reactive oxygen species (ROS) production,

intracellular glutathione (GSH) depletion, and lipid peroxidation in LPS treated cells. The results indicate that the extract and rosmarinic acid are able to suppress LPS-induced biological changes in gingival fibroblasts (Zdarilov *et al.*, 2009).

For the *in vivo* study about the antioxidant properties of the *S. prunellae,* the antioxidant activities of polyphenolic extract from *Prunella vulgaris* in rat suggesting mainly sparing effect on erythrocyte reduced glutathione, an important component of endogenous antioxidant system (Skottov *et al.*, 2001).

Further studies conducted by Skottov *et al.*, (2004) shown that the phenolics-rich extracts of *S. prunellae* improved the antioxidant status in blood and liver and positively affected plasma lipoprotein profile in an experimental model of dietary induced hypertriglyceridemia using the rat model (Skottov *et al.*, 2004).

*S. prunellae* not only possesses antioxidant property but also the antiviral effect. An anionic polysaccharide was isolated from *S.prunella* by hot water extraction, ethanol precipitation and gel permeation column chromatography. This polysaccharide at 100 mg/ mL was active against the herpes simplex virus types 1 and 2 (HSV-1 and HSV-2). The polysaccharide isolated from *P. vulgaris* has specific activity against HSV and its mode of action appears to be different from other anionic carbohydrates, such as heparin (Xu *et al.*, 1999). Furthermore, a polysaccharide fraction from *S. prunellae* effects on the expressions of HSV-1 and HSV-2 antigens in their host Vero cells were investigated. The results show that *S.prunellea* is effective

against both the HSV-1 and HSV-2 infections, and flow cytometry offers a quantitative and highly reproducible anti-HSV drug-susceptibility assay (Chi-Ming Chiu *et al.*, 2004). The recent study regarding this polysaccharide, there was a lignin–carbohydrate complex, PPS-2b (MW= 8500) that was isolated from *S. prunellae* by ethanol precipitation, dialysis, CTAB precipitation, and gel exclusion chromatography and analysed by HPLC. This complex has shown an anti-HSV activity in plaque reduction assay (Zhang *et al.*, 2007).

Besides the antiviral activity toward HSV, it has shown inhibitory and antiviral activity toward a component of small molecular organic compounds in HIV1. The results suggest that tannin may be one of major inhibitors of the HIV-1 gp41 six-helix bundle formation in the herb extracts and that tannin may inhibit HIV- 1 entry by disrupting the gp41 six-helix bundle formation (Liu *et al.*, 2002).

From the immunological aspect, the immunosuppressive activity of the ethanol extract of *S. prunellae* consisting of a mixture of triterpenoids, flavonoids, tannins and polysaccharide was studied on the immune responses in mice. The results suggest that ethanol extract of *S. prunellae* could suppress the cellular and humoral response in mice (Sun *et al.*, 2005).

The effects of an aqueous extract of *S. prunellae* on immunostimulatory and antitumor activity of *S. prunellae* in murine macrophage RAW 264.7 cells were tested. *S. prunellae* extract stimulated macrophage phagocytic activity, nitric oxide (NO) production and cytostatic activity. It also induced the gene

expression and production of macrophage-related cytokines such as TNF-a, IL-1b and IL-6 (Han *et al.*, 2009).

A cyclodextrin-modified capillary zone electrophoresis method was successfully applied to determine ursolic acid, oleanolic acid and betulinic acid, caffeic acid, *p*-coumaric acid, rosmarinic acid, rutin and quercetin in the samples of *S. prunellae* and its beverage drink products. Only the isomeric compounds and rosmarinic acid could be detected in the *S. prunellae* while only rosmarinic acid was detected in the beverage products (Cheung & Zhang, 2008). Recently, a HPLC-UV technique was employed to study about this herb recently and five triterpenic acids were separated from *S. prunellae* crude ethanol extract as marker compounds for use in the quality control of herbal medicines (Lee *et al.*, 2009).

## 2.2 Pericarpium of the *C. reticulatae*

Previous studies shown that the orange peel consists of essential oil, with d-limoneme, citrol, isopropenyltoluoene, delta-elemene, alpha-humulene, beta-sesquiphol-landrene, alpha-humulenol acetate, and 1, 8 menthadien-10-ol-acetate. Besides, it also contains flavonoids and other components of hesperidin, carotene, crypto santhin, vitamin B, C, and P, alkaloid synephrine, and N-methyltyramine (Hou & Jin, 2005).

As essential oil is concerned, there was a comparative study has been conducted to investigate the similarities and differences of essential oil components in pericarpium of *C. reticulatae viride* and *C. reticulatae* by using GC–MS combined with alternative moving window factor analysis (AMWFA). About 59 compounds in the essential oils of pericarpium of *C. reticulatae* were identified. Main compound was d-limonene that accounted for 65.61–83.14% (Wang *et al.*, 2008).

Regarding the flavonoids substance in the pericarpium of *C. reticulatae*, the polymethoxylated flavones were extracted from pericarpium of *C. Reticulatae Viride* by using a procedure that obtained a consistent mixture of PMFs both in identity and proportion. The mixture was consisted of isosinensetin, sinensetin, tetramethyl-*o*-isoscutellarein, nobiletin, tetramethyl-*o*-scutellarein, tangeretin, 5-demethylnobiletin, 5-demethyl tangeretin and other flavonoids including heptamethoxyflavone (Wang *et al.*, 2007).

In another study, flavonoid extract is obtained from pericarpium of *C. reticulatae* using 80% aq. ethanol as solvent to determine the antioxidant and antimicrobial activity. By the HPLC analysis, hesperidin, nobiletin and tangeretin were separated and determined. It was found that the hesperidin displayed a broad antimicrobial spectrum and exerted antimicrobial effects in antimicrobial tests and the properties of antimicrobial effect in the flavoniods extract may mostly contributed by hesperdin (Yi *et al.*, 2008).

Not only the antimicrobial effect, the polymethoxyflavones in the peels of *C. reticulatae* also identified and further herb suggested that it was excellent sources of functional polymethoxyflavones that may help prevent female cancers, such as ovarian and breast cancer. Note that the polymethoxyflavones of the herbs was identified as isosinensetin, sinensetin, nobiletin and tetramethyl-o-scutellarein by a combined separation using high-speed countercurrent chromatography and preparative high performance liquid chromatography, and structure elucidation by electrospray ionisation mass spectrometry (ESI-MS) and 1H and 13C nuclear magnetic resonance (NMR) (Du & Chen, 2009).

The pericarpium of *C. reticulatae* has various type of effects on human, many of the research was done for proving those effects of the orange peel and further discover the potential value of the herb. Most of the research using the crude extract without identified the compounds that actually obtained from the extract.

The anti-cancer property also had been determined by using the crude extract of the *C. reticulatae.* The result shown that the expression of pro-apoptotic gene, *Bax*, was increased and the expression of anti-apoptotic gene, *Bcl-2*, was decreased by *C. reticulatae* extract treatment. The expression and activity of major apoptotic gene, *caspase-3* was significantly increased by *C. reticulatae* extract treatment. *C. reticulatae* extract could induce the apoptosis on SNU-C4, human colon cancer cells via Bax-related caspase-3 activation (Kang *et al.*, 2005).

In a study to determine the antioxidant effect of the citrus herbal products, the total phenolic content, DPPH free radical-scavenging activity, hydrogen peroxide-scavenging activity, ferrous ion-chelating activity and ferric-reducing antioxidant power (FRAP) of *C. reticulatae* extracts were determined. The antioxidant activities of four pericarpiums of *C. reticulatae* was found in the medium but failed to give a very good reducing power (Su *et al.*, 2008). This may due to the effect of the other component in the extract which lowers the antioxidant action.

There was a study examined how *Citrus* herbal extract affect the differentiation of 3T3-L1 adipocytes. These results suggest that dietary pericarpium of *C. reticulatae* suppresses 3T3-L1 differentiation by down-regulation of adipogenic transcription factors. Experimental data may prove useful in further medical examination of the use of orange peel for body weight control (Sheu *et al.*, 2007).

However, there was also some new method developed to study on the specific flavoniods compounds, especially hesperidin and synephrine. A method based on capillary electrophoresis with electrochemical detection (CE–ED) has been developed for the determination of hesperidin and synephrine in the pericarpium of *C. reticulatae*. These two analytes were well separated within 5 min in a 40 cm long capillary at a separation voltage of 12 kV in 50 mmol/ L borate buffer (pH 9.0) from pericarpium of *C. reticulatae* (Gang Chen, 2002). Besides, there is also another new method developed for determination of these two compounds in the orange peel. These two compound was determined by using a new, simple, and fast method which integrate indium tin oxide electrode in an amperometric detection (AD) microchip (Wang *et al.*, 2006). From the chromatography aspect, that is also a new ion pairing chromatographic method was developed to exclude synephrine from the viod volume and to maintain selectivity between the hesperidin and naringin, which the contents of hesperidin, naringin and synephrine in several *Citrus* herbs were simultaneously determined by this new method (Ding *et al.*, 2007).

Some of the previous HPLC study about this herb mainly using the methanol extract as the sample. For instance, hesperidin contents in pericarpium of *C. reticulatae* was performed by a simple extraction with methanol and semi-micro HPLC with electrochemical detection (μHPLC–ECD) (Xia *et al.*, 2006).

A potential study for quality control of traditional Chinese medicine by using HPLCalso using the methanol extract as the sample with the hesperidin,